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DEPRESSED LUTEINIZING HORMONE RESPONSE TO ESTRADIOL IN VIVO AND GONADOTROPIN-RELEASING HORMONE IN VITRO IN EXPERIMENTALLY DIABETIC SWINE[1]

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The influence of the acute withdrawal of insulin therapy in streptozocin-diabetic female swine was examined for changes in 1) the in vivo pulsatile secretion of luteinizing hormone (LH), 2) the preovulatory-like gonadotropin patterns after exogenous estradiol, and 3) the in vitro LH secretion by cultured pituitary cells. In Experiment 1, ovariectomized diabetic pigs (n = 4) were maintained with insulin therapy until 4 d before estradiol benzoate (EB; 7 μg/kg body weight; subcutaneous) was administered. Four normal ovariectomized pigs, matched for age and weight, served as controls. The diabetic state was confirmed by the measurement of glucose and insulin concentrations during a glucose tolerance test. Pulsatile LH secretion was not influenced by experimental diabetes mellitus. However, the expected surge in LH was not induced by EB in diabetic gilts. In contrast, three of four normal gilts had a preovulatory-type surge in LH. Concentrations of follicle-stimulating hormone in serum were not affected by diabetes mellitus. Estradiol concentrations in serum after EB were influenced by diabetes mellitus (treatment by time interaction; $P < 0.001$). In individual estradiol profiles, maximum concentrations were similar (104 ± 10.4 and 91 ± 12.0 ng/ml for normal and diabetic pigs, respectively), but the interval to maximum concentration was delayed in diabetic pigs (27.5 vs. 9.0 h; SE = 3.0; $P < 0.05$). However, the duration of standing estrus (2.2 ± .3 d) and the interval from EB to estrus (3.6 ± 0.3 d) were not influenced by diabetes mellitus. In Experiment 2, LH secretion by cultured cells and residual cellular LH content were greater in the pituitaries of normal than diabetic pigs ($P < 0.05$), and only cells from normal pigs responded to gonadotropin-releasing hormone (GnRH), with increased production of LH ($P < 0.05$). In conclusion, diabetes mellitus did not affect pulsatile LH secretion but did lower the ability of exogenous estradiol to stimulate a surge in vivo and of GnRH to increase LH in vitro, suggesting that the pituitary response to estradiol and GnRH is more severely affected by diabetes than is the GnRH pulse generator.

INTRODUCTION

Diabetes mellitus is associated with reproductive dysfunction in humans (1,2), and the experimentally induced disease is associated with a failure of estrous cyclicity in rats (3–6) and pigs (7). Coordinated function of the hypothalamo-pituitary-ovarian axis is impaired in diabetes mellitus, and certain components of this impairment have been

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identified. In the rat, the absence of ovulation in diabetes was attributed to the failure of the positive feedback of estradiol on gonadotropin-releasing hormone (GnRH) (4,8) and reduced sensitivity of the pituitary to GnRH (5,9,10). With respect to circulating luteinizing hormone (LH) concentrations, diabetic rats were more sensitive to the negative feedback of steroids on LH (11) and less sensitive to the positive feedback of estradiol and progesterone on LH (10,11). A positive role for insulin to increase gonadotropin secretion was demonstrated in cultured pituitary cells (12).

The effects of diabetes mellitus on hypothalamic GnRH depend on the duration of the untreated condition. Experimental diabetes mellitus in rats for 10 d did not affect hypothalamic GnRH release in vitro (11), but longer term diabetes (>30 d) was associated with lesions in hypothalamic GnRH-producing neurons (13). On the basis of pituitary LH secretion in response to GnRH, Blades et al. (10) observed that 3 d of untreated diabetes mellitus impairs the feedback responsiveness of LH to steroids, suggesting that ovarian feedback is affected before GnRH production itself is altered.

The withdrawal of insulin therapy from cyclic diabetic swine during Days 12 through 18 of the estrous cycle resulted in reduced ovarian follicular development and increased atresia, accompanied by lowered intrafollicular concentrations of estradiol and insulin-like growth factor-I (14). However, in that study, average concentrations of LH were unchanged by diabetes mellitus during Days 12–18 of the estrous cycle and the pulse frequency of LH increased in diabetic animals. These data suggest that impaired follicular function could not be attributed to reduced gonadotropins, but the presence of the ovary prevented the direct assessment of the effects of diabetes on gonadotropins.

The objective of the experiments presented here was to examine the characteristics of the hypothalamo-pituitary axis in the experimentally diabetic pig. In Experiment 1, we examined the in vivo effects of experimental diabetes mellitus on pulsatile LH secretion and on the estradiol-induced preovulatory LH surge in ovariectomized animals. In Experiment 2, we examined in vitro LH secretion in response to GnRH by cultured pituitary cells removed from those animals.

MATERIALS AND METHODS

Animals and Sampling. *Experiment 1.* A total of eight 10-mo-old crossbred gilts, four streptozocin-induced (induced at 8–12 wk of age) diabetic gilts (weight, 203 ± 7 kg) and four normoglycemic controls (211 ± 8 kg), were ovariectomized at least 30 d before the determination of pulsatile LH secretion and the response of LH and follicle-stimulating hormone (FSH) secretion to estradiol benzoate (EB). The diabetic gilts were maintained on daily porcine insulin suspension (Lente Purified Pork; Squibb-Novo, Inc., Princeton, NJ; subcutaneously) until 4 d before the experiment began, glucose levels were monitored twice weekly with Dextrostix (Miles, Inc., Elkhart, IN) and a portable glucose meter, and the gilts were given full access to feed, as we have previously reported (13). Insulin dosages were adjusted so that blood glucose concentrations were between 50 and 100 mg/100 ml at 3 hr after insulin injection, which represented dosages ranging from 0.70 to 1.5 IU/kg. This insulin replacement regimen is supportive of normal ovarian function (7,13,15). All diabetic pigs were allowed full access to feed (maintenance diet of corn and soybean meal, 16% crude protein) and water and were weighed weekly to monitor weight change. Normal pigs were fed to have similar weight gains (approximately 90% of ad libitum). All pigs were exposed to natural photoperiod and temperature in July (33.5° N, 88° W). Analyses of concentrations of growth hormone and insulin-like growth factor-I from these animals have been reported elsewhere (16).

Insulin therapy was terminated 4 d before EB was administered. On the last day of insulin treatment, the pigs were weighed and placed in individual pens where they were

housed for the duration of the study. Polyethylene catheters were inserted nonsurgically and aseptically into the anterior vena cava. On the day of EB administration (7 μg/kg in sesame oil; 200 μg/ml; intramuscularly), samples were obtained at 10-min intervals for 4 hr before EB (0800–1200); then, sampling continued at 10-min intervals for a second 4-hr period after EB (1210–1600). Thereafter, samples were obtained every 6 hr through 114 hr, except that frequency was increased to every 2 hr from 66 through 102 hr in order to characterize the anticipated LH surge. One diabetic pig lost patency of the catheter after 96 hr of sampling and was missing from analyses after that time. The pigs were observed daily for visual signs of estrus-like behavior—vulva color and swelling and standing to application of back pressure. To verify the existence of diabetes, at the end of the study, a glucose challenge consisting of an intravenous infusion of 50% dextrose (500 mg/kg body weight) was administered. Blood samples were obtained at 0, 2, 4, 6, 8, 10, 20, 30, 40, 60, and 120 min to determine glucose clearance values. Glucose clearance rates (K) were determined quantitatively according to the formula log(glu1)-log (glu2)/(time1–time2), with time1 being the time of the highest glucose concentration (glu1) postinfusion and time2 being the time of the lowest glucose concentration (glu2) postinfusion (17).

Experiment 2. The same pigs as in Experiment 1 were used in a second experiment. After insulin therapy was reinitiated (as described above) for 4 mo, animals had insulin therapy removed for 7 d, after which they were euthanatized by electrical stunning, followed by exsanguination. Although gilts were removed from insulin therapy for 8 d total in Experiment 1, it was considered that 7 d was the maximum time gilts should be maintained without insulin, on the basis of signs of lethargy. Body weights were 227 ± 9 and 214 ± 8 kg for normal and diabetic pigs, respectively. Pituitary glands were aseptically removed from gilts at the time of slaughter. All subsequent procedures were performed under sterile conditions. The anterior lobe was dissected from each pituitary gland, and cells were enzymatically dispersed and cultured as previously described (17). Cell viability and number were determined by counting the number of cells excluding trypan blue on a hemocytometer. Cells were diluted on the basis of the number of live cells to 8×10^4 cells/ml with culture medium (Dulbecco's modified Eagle's medium [DME] and Ham's Nutrient Mixture F-12 [F-12] + 10% fetal bovine serum [Sigma Chemical Co., St. Louis, MO]) containing 100 mg of glucose/100 ml, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 250 ng/ml of amphotericin B (Sigma). Cells were plated at 1 ml of cell suspension per well in a 24-well cluster plate (day of seeding = Day 0 of culture) and cultured at 37° C in a humidified atmosphere containing 5% CO_2. Culture medium was changed 48 hr after seeding, and experiments were performed on Day 4. On Day 4, old medium was discarded, and plates were rinsed twice with serum-free DME/F-12 containing 0 (control), 10^{-9}, 10^{-8}, or 10^{-7} M GnRH (three to six wells per treatment per pig). Medium was harvested at 4 hr, and cells were then solubilized with 0.2% Triton X-100 to recover intracellular LH.

Assays. Serum (19,20) and culture medium concentrations and cellular contents (21) of LH and serum concentrations of FSH (20) were measured by validated radioimmunoassays (RIAs). Intra-assay and interassay coefficients of variation (CV) for the LH RIA for serum were 8 and 14% (mean of two assays) respectively, and the sensitivity of the assay was 0.30 ng/ml. All samples from cell cultures were measured in one assay; the intra-assay CV was 8%, and the sensitivity was 0.30 ng/ml. FSH intra-assay and interassay CV were 9 and 14%, respectively, and the sensitivity of the assay was 0.40 ng/ml.

Estradiol in serum was assayed by procedures validated in our laboratory (22). Intra-assay and interassay CV were 8 and 11%, respectively, and the sensitivity of the assay was 0.8 pg/ml. The cross-reactivity of antibody with EB was determined to be 1.5%. Testos-

terone was assayed by procedures validated in our laboratory for serum (12). Intra-assay and interassay CV were 9 and 14%, respectively, and the sensitivity of the assay was 0.02 ng/ml. Cortisol was assayed by a commercial double-antibody RIA kit (DSL 2000; Diagnostic Systems Laboratories Inc., Webster, TX). All samples were measured in one assay, and the intra-assay CV was 5.2%.

Serum insulin concentrations were determined by the use of a double-antibody RIA procedure validated in our laboratory (20). The intra-assay and interassay CV were 10.7 and 11.2%, respectively. The sensitivity of the assay was 0.04 ng/ml. For the quantitative determination of glucose in plasma, the glucose oxidase method was used (20).

Statistical Analysis. Hormone and metabolite concentrations in serum were analyzed by analysis of variance with a general linear model for split plot with the main effect being glycemic state (insulin-dependent diabetes or normoglycemia); the whole-plot error term was animal within glycemic state, and the subplot was time (23). Individual hormonal profiles were examined for the interval to maximal suppression and the LH surge after EB injection. The existence of the LH surge was determined by the use of the following criteria: 1) time of emergence is the time when serum LH concentrations are equal to or more than three standard deviations above mean baseline concentrations (baseline = mean of first 48 hr of sampling) and is followed by increasing serum LH concentrations in the subsequent sample; 2) the peak magnitude is at least 50% higher than the serum LH concentration at Time 0; 3) the termination of the LH surge is when the serum LH concentration is equal to that of Time 0; and 4) the time from the emergence to the termination of a surge is at least 20 hr. One-way analysis of variance for these variables and for LH pulse characteristics, determined by the criteria of Goodman and Karsch (24), was used. Basal secretion or the content of LH produced by pituitary cells was defined as the amount of LH per 8×10^4 cells secreted into the culture medium or contained in cells in the absence of GnRH. Data obtained from each pituitary gland constituted a single animal replicate and were subjected to one-way analysis of variance to test the effects of diabetes mellitus (23).

RESULTS

Pulsatile LH (Experiment 1). In Experiment 1, pulsatile LH secretion was similar in normal and diabetic pigs after 4 d without insulin (Table 1). All characteristics of pulsatile LH secretion were similar before and after EB (data not shown). Concentrations of LH during 114 hr of sampling after EB were similar (data not shown). However, an analysis of each pig's individual hormone profiles revealed that three of four normal pigs exhibited preovulatory LH surges (Figure 1), whereas no diabetic pigs met the criteria for a preovulatory surge (Figure 2). The suppression of LH by EB occurred in both groups, but in the diabetic pigs, LH rebounded to pretreatment concentrations. These rebounds were at the same time as the LH surge (60–72 hr after EB) that occurred in normal pigs. The maximum serum concentrations of LH reached were similar for normal and diabetic pigs

TABLE 1. MEAN AND BASELINE LH CONCENTRATIONS, LH PEAK FREQUENCY, AND LH PULSE AMPLITUDE FOR DIABETIC AND NORMAL GILTS FOR 8 HR.

Item	Diabetic	Normal	SE
Number of Pigs	4	4	
Peak Number	8.5	8.8	1.5
Peak Amplitude (ng/ml)	0.8	0.9	0.2
Baseline LH (ng/ml)	1.2	1.2	0.3
Average (ng/ml)	1.5	1.5	0.3

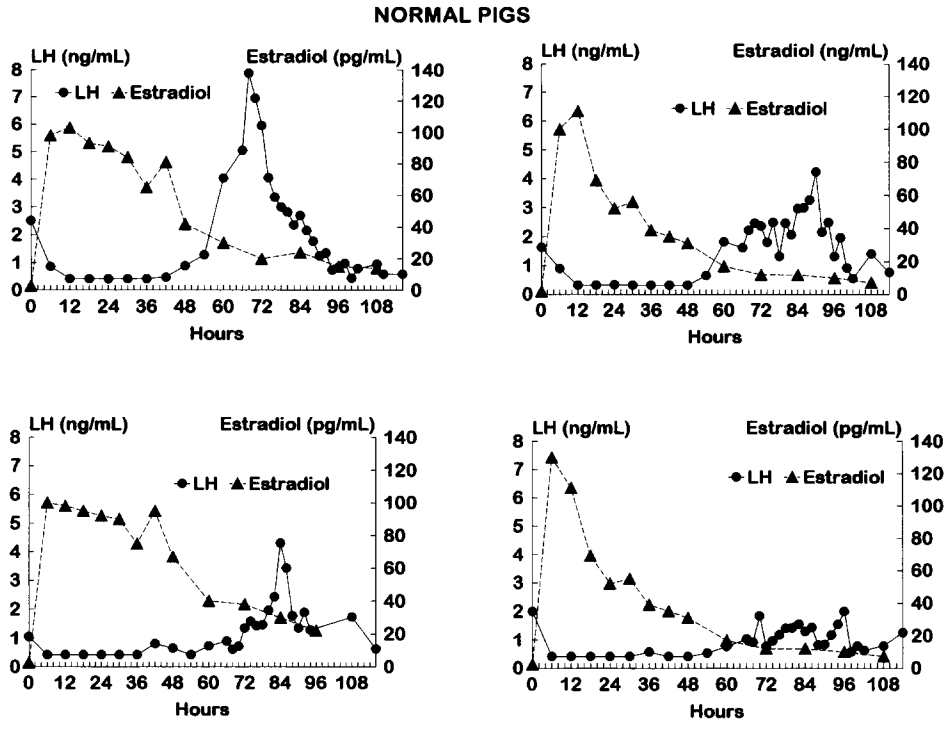

Figure 1. Individual profiles of LH and estradiol in normal pigs during 114 hr after EB injection. A preovulatory surge of LH was detected in three pigs (all except lower right panel).

(2.0 ± 1.0 and 4.4 ± 1.1 ng/ml, respectively). FSH concentrations during 114 hr after EB averaged 3.6 ± 0.9 ng/ml, were not influenced by diabetes, and were similar before and after EB (data not shown).

Steroid Concentrations. In individual hormone profiles (Figures 1 and 2), glycemic state did not affect maximal estradiol concentrations (104 ± 10.4 and 91 ± 12.0 pg/ml for normal and diabetic pigs, respectively). However, an analysis of individual estradiol profiles revealed that the interval to the maximal estradiol concentration was delayed in diabetic pigs (27.5 vs. 9.0 hr; SE = 3.0; $P < 0.05$). Serum estradiol patterns during 114 hr after EB were influenced by diabetes mellitus (treatment by time interaction, $P < 0.001$; Figure 3). By 114 hr after injection, values in normal animals returned to pretreatment levels. In contrast, estradiol in diabetic pigs declined by 48 hr after EB but remained elevated above pretreatment concentrations for the remainder of the sampling period (Figure 3). Testosterone concentrations increased during the sampling period in diabetic pigs and were higher (0.06 ± 0.004 ng/ml; $P < 0.01$) than those in normal pigs, which had values below the sensitivity of the assay (0.02 ng/ml). Mean concentrations of cortisol were similar ($P = 0.10$; 32 ± 9.5 vs. 14 ± 4.9 ng/ml for diabetic and normal pigs, respectively).

Estrus and Body Weight. In Experiment 1, diabetic pigs lost 3.3 ± 0.04% of their body weight over the 9-d period without insulin therapy, which was similar to the body weight change of normal gilts (–6.7 and 0.0 kg, respectively; SE = 2.4; $P = 0.10$). Diabetic pigs had normal estrus-like behavior, exhibiting onset 3.7 ± 0.3 d after EB, compared with 3.5 ± 0.3 d after EB for normal animals. The duration of standing estrus was similar for both groups (2.0 ± 0.3 and 2.3 ± 0.3 d for diabetic and normal pigs, respectively).

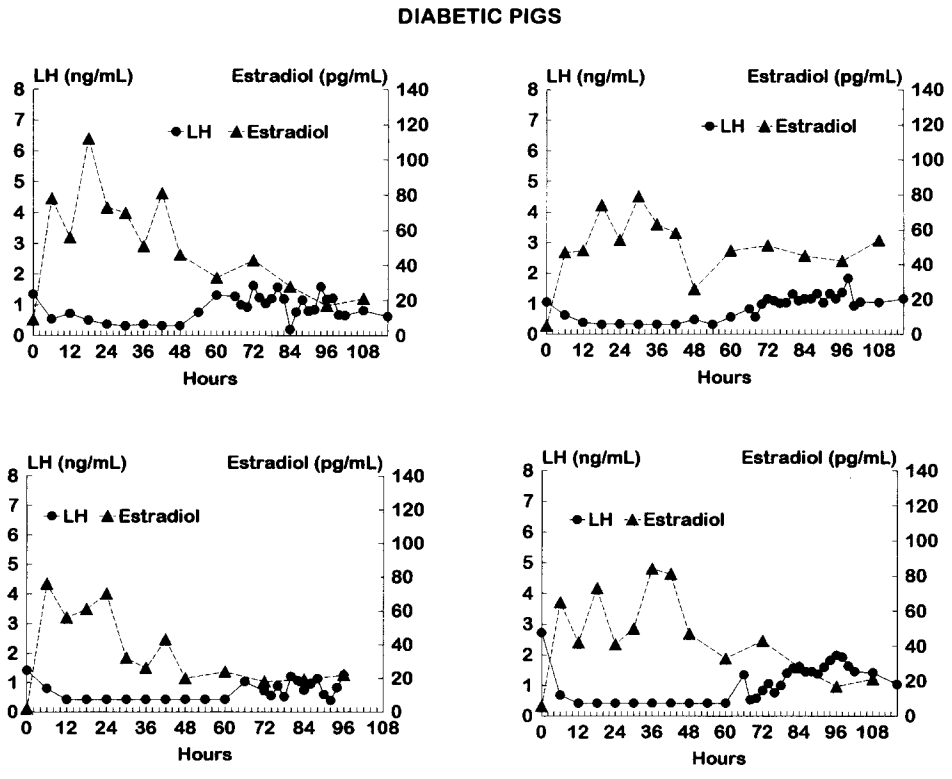

Figure 2. Individual profiles of LH and estradiol in diabetic pigs during 114 hr after EB injection. Preovulatory surges of LH were not detected in any pigs. Note that the same scale was used as in Figure 1, to allow for comparison between diabetic and normal pigs.

Verification of Diabetic State. In Experiment 1 at the time of EB injection (after 4 d without insulin), plasma glucose concentrations were 545 ± 10 and 82 ± 5 mg/100 ml and serum insulin concentrations were 0.5 ± 0.1 and 1.5 ± 0.3 ng/ml in diabetic and normal pigs, respectively. After the glucose challenge, serum insulin concentrations were lower in diabetic than in normal pigs (maximum values of 0.32 and 3.02 ng/ml; SE = 0.16; $P < 0.0001$). Overall mean glucose concentrations during the 120-min period of the glucose tolerance test were higher in the diabetic than in the normal pigs (482.4 and 113.8 mg/100 ml; SE = 45.8; $P < 0.001$). The glucose clearance rate (K) was lower in diabetic than in normal pigs (0.20 vs. 2.28; SE = 0.48; $P < 0.02$).

Experiment 2. In Experiment 2, fasting serum insulin concentration was lowered ($P < 0.05$; 0.6 vs. 1.7 ng/ml; SE = 0.3) and glucose was elevated in diabetic pigs ($P < 0.05$; 377 vs. 108 mg/100 ml; SE = 16) at the time of euthanasia. The number of pituitary cells recovered was higher for diabetic pigs ($2.2 \pm 0.07 \times 10^6$ vs. $1.3 \pm 0.12 \times 10^6$ cells per pituitary; $P < 0.05$). Figure 4 illustrates LH production and residual cellular content in response to GnRH. Basal LH secretion and LH content from the pituitary cells of diabetic animals was lower ($P < 0.05$) than those for normal animals, whereas the LH response to GnRH was absent for pituitary cells from diabetic animals.

DISCUSSION

Pulsatile patterns of LH were not affected by glycemic state, and FSH was not affected by EB or glycemic state. If pulsatile LH reflects GnRH secretion (25), then we infer that

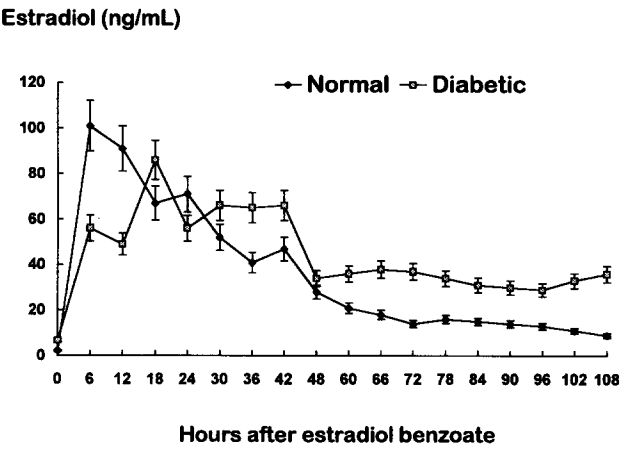

Figure 3. Least-squares means and SE (vertical bars) of estradiol after a subcutaneous injection of EB for diabetic and normal pigs (n = 4 each).

hourly GnRH secretion also was not affected by experimental diabetes mellitus. Similarly, Spindler-Vomachka and Johnson (11) reported no differences in hypothalamic GnRH output in vitro from rats in which experimental diabetes existed for 1 wk or less. However, hypothalamic GnRH content as well as neurosecretory cell integrity was reduced in other studies of longer term untreated diabetes mellitus in rats (6,26,27). In contrast to the results presented here, ovary-intact pigs with diabetes mellitus for 4 d during the estrous cycle had elevated pulsatile LH secretion compared with normal pigs (13). We observed earlier that serum growth hormone concentrations were dramatically elevated after 4 d without insulin therapy (16). Together, these results suggest that in short-term untreated diabetes mellitus in swine, there are other changes that precede negative effects on the hypothalamic GnRH pulse generator.

In this study, the estradiol-induced LH surge was absent in the diabetic pig, and LH patterns indicated that positive (failure of LH surge) feedback by estradiol was abnormal. In previous studies performed in ovariectomized pigs, LH concentrations were elevated after ovariectomy, and EB induced a suppression in LH and hypothalamic GnRH that was followed by a surge in LH (21,28,29). The ability of EB to induce a rapid suppression of LH indicates that the sensitivity to the negative feedback effects of estradiol in diabetic pigs was equivalent to that of normal pigs. The failure of positive feedback by estradiol on LH in diabetic pigs could be explained by a number of factors including insufficient hypothalamic GnRH production, failure of the pituitary to respond to GnRH and/or estradiol, or defects in receptor action or signal transduction. The fact that less LH was produced by cultured pituitary cells of diabetic pigs supports the concept that insufficient LH is produced during diabetes to produce a preovulatory-like surge in LH.

The finding that diabetic animals were less sensitive to both positive and negative aspects of gonadal steroids has been reported in rats (10,11). Distiller et al. (31) suggested that glucose metabolism plays a major role in gonadotropic function, because there was an inverse relationship between fasting glucose level and LH. In addition, glucose infusion suppressed the LH response to GnRH in the prepuberal pig (32). Collectively, these data indicate that altered glucose metabolism precipitated by the absence of sufficient insulin could inhibit LH secretion in response to estradiol and endogenous GnRH.

Delays both in reaching maximal serum estradiol concentrations and in returning to basal concentrations in diabetic pigs may be partially explained by a decrease in the rate

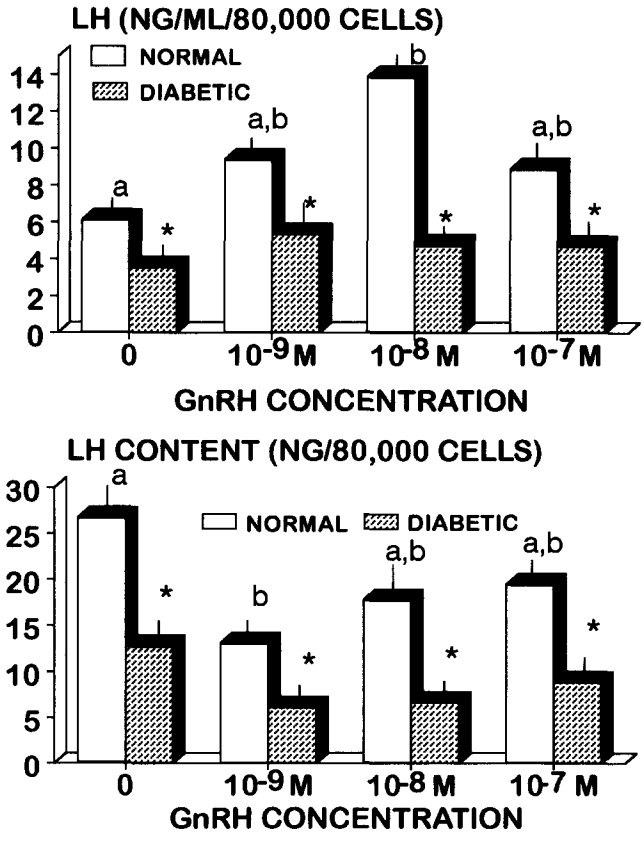

Figure 4. Least-squares means and SE (vertical bars) of LH production (top panel) and cellular content (bottom panel) in normal and diabetic pigs (n = 4 each). Asterisks indicate significant differences ($P < 0.05$) between diabetic and normal animals. Means with different letters differ ($P < 0.05$) for normal pigs only.

of peripheral circulation. Although not measured, lowered ear temperature was noted in the diabetic pigs, which could indicate decreased blood flow and a more gradual absorption of EB into the circulation. However, all pigs exhibited a similar estrus response. In contrast, insulin withdrawal from diabetic rats decreased estrogen-induced sexual receptivity (33,34). In those studies, circulating estrogen concentrations were not reported. In this study, the fact that characteristics of estrus were normal, yet LH surge patterns were abnormal in the diabetic pig, indicates that estrogen-responsive brain centers controlling estrus and GnRH secretion could have different sensitivities to the amount or pattern of estradiol.

In Experiment 2, lowered LH production by the pituitary cells of diabetic pigs persisted after 3 d in culture, and LH did not increase when GnRH was added. Thus, both basal and GnRH-induced LH secretory capacities appear to be impaired as a consequence of diabetes. The lowered LH production by cells from diabetic gilts is somewhat in contrast to the results of Experiment 1, in which pulsatile LH secretion was not affected by diabetes after 4 d without insulin therapy and in which LH concentrations were similar after four more days, although a surge was not induced in diabetic pigs. As noted earlier, more cells were harvested from the pituitaries of diabetic than normal pigs. If those cells contained a disproportionate number of nongonadotropic cells, it could explain why LH production was lower than that in normal pigs. However, if gonadotropes functioned normally, a

response to GnRH, albeit at a lower concentration, would still be expected. The type of cells accounting for the increased cell number was not identified, but it has been noted that growth hormone production is increased in diabetic pigs, suggesting that somatotrope numbers could be increased in diabetic compared with normal pigs (16).

The diabetic animals had higher circulating testosterone than the normal animals, although concentrations were low. In female rats, elevated testosterone in diabetic animals was not apparent after adrenalectomy, suggesting an adrenal origin (35,36). Elevations in testosterone similar to those found in this study were observed in ovary-intact diabetic pigs, which also had elevated concentrations of nonesterified fatty acids (7). However, in that study, circulating LH was not decreased by diabetes mellitus (7), suggesting that testosterone was not exerting negative feedback. The authors suggested that dysfunction of adipose tissue metabolism and possible lowered aromatase activity in adipose tissue could explain the significant elevation in testosterone in the diabetic gilt. Moreover, the differences in serum estradiol concentrations after EB treatment may be in part related to adipose tissue function. To further explore differences in adrenal function, cortisol was assessed, and a tendency ($P = 0.10$) for higher concentrations was observed in the diabetic compared to the normal pigs, also indicative of altered adrenal function.

Weight loss has been reported to affect gonadotropic function. In Experiment 1, diabetic pigs lost 3.3% of their body weight over the 9-d study. Weight loss sufficient to cause cessation of estrous cycles in pigs was 14.5% of initial body weight (37). Moreover, weight loss comparable to that in diabetes mellitus did not alter the ability of rats to have an LH surge (5). Therefore, we conclude that differences in weight loss did not account for the influences on the LH surge observed in this experiment.

The acute withdrawal of insulin from experimentally diabetic pigs under the conditions of this study did not influence pulsatile LH secretion. This result agrees with those from our earlier studies, which indicate that in short-term untreated diabetes mellitus, the ovary produces fewer steroids even though circulating LH is not affected (7,13). However, the preovulatory surge mechanism appears particularly sensitive to untreated diabetes mellitus. The in vivo results suggest that diabetes mellitus prevents the preovulatory surge by altering the sensitivity of the hypothalamus and/or pituitary to estradiol or the sensitivity of the pituitary to endogenous GnRH. The cell culture experiments confirm that the sensitivity of the pituitary to GnRH is altered by diabetes mellitus. Although circulating estradiol had a more gradual increase in diabetic pigs, these differences did not prevent behavioral responses to estradiol that were identical to those of normal animals. However, differences in circulating steroids cannot be ruled out as an explanation for the failure of diabetic pigs to have a preovulatory-like surge in LH. We conclude that diabetes mellitus did not affect pulsatile LH secretion, providing indirect evidence that the hypothalamic GnRH pulse generator was not affected by diabetes. However, the ability of estradiol to stimulate a preovulatory-like LH surge in vivo and of GnRH to increase LH release in vitro is compromised by diabetes mellitus, suggesting that gonadotrope sensitivity to hormonal stimuli is affected by the consequences of metabolic hormonal alterations.

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